

Ph.D. Thesis

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## Investigation of trehalose metabolism in fungal and plant species

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## **I. Introduction**

Extreme weather conditions and swift climatic changes, caused by the global warming, set serious demands for the wildlife. Abiotic environmental parameters continuously change in nature, however to variable extent; when they reach values out of the optimum range of the species (water deficiency, extreme temperature values, osmotic load, unfavourable light conditions or unbalanced mineral nutrition) that means a stress situation for the living organisms. Adaptation to these circumstances, enabled by different defence mechanisms, is crucial for the survival.

According to the predictions, one of the most serious future problems is the unfavourable change in the precipitation, which may also considerably affect the agricultural regions of Hungary: the total area of crop fields involved at least temporarily in drought stress is expected to expand. On the meantime, the intemperately elevated precipitation level may cause serious invasions of the parasites.

The stress caused by water deficit is one of the most serious challenges in terms of preserving homeostasis. Maintaining osmotic balance is essential for the organisms, thus among unfavourable conditions, different defence mechanisms are activated.

In case of microorganisms and fungi a well-known response is the synthesis and accumulation of bio-protective compounds. If the stress becomes more serious, they produce spores and resting bodies, and they survive the harsh period in dormancy.

Development, growth and productivity of sessile, terrestrial plants are simultaneously affected by several environmental factors; they can tolerate the stress effects to different extent, depending on their potential on the individual, population and the species level. Deleterious effects of drought seriously affect plants; species of the very same habitat may be considerably different concerning their tolerance. Several effective strategies have evolved, e.g. the regulation of transpiration, metabolic and structural changes or the development of extreme anhydrobiotic state. Accumulation of osmoprotective compounds is characteristic of species with considerable or extremely high tolerance. In case of reproductive tolerance, notable is the establishment of the sucrose-based biological vitreous state as a result of water loss, what ensures the survival of the pollen grains or the embryo.

Bioprotective compounds are characteristically small molecules, and their special features enable them to prevent the negative effects of the stressors, or to reduce the extent of the injuries as much as possible by stabilizing the structure of the macromolecules.

Trehalose is the most efficient bioprotective molecule of nature. Its outstanding characteristics derive from its special physico-chemical features. During water loss, it can

replace water molecules that stabilize the structure of biological molecules (membrane lipids, proteins). As a successful biological vitreous-forming molecule, it enables the re-establishment of the original molecular structure during rehydration, when circumstances become favourable again.

Discovering the genetic background, expression pattern and activity regulation of the enzymes of trehalose synthesis and accumulation, both among bacteria and eukaryotes, is significant for both basic and applied research. For instance, it would empower us to increase the stress tolerance of industrial plants by transforming the genes of trehalose metabolism.

My PhD works have focused on trehalose metabolism. My results on *Morchella* species can confirm and evidence the models describing the ontogeny of this ascomycetous genus of special life cycle. The experiments with *Populus* species, being of ecological, industrial and nature conservational significance, contribute to the results of this scientific field in higher plants, and aid to clarify the role of trehalose in the plant metabolism.

## II. Aims

- demonstrating the inductive role of oxidative stress in the formation of resting bodies (pseudosclerotium, PS) in *Morchella steppicola*, and revealing correlations between oxidative stress, PS formation, trehalose synthesis and accumulation
- demonstrating the bioprotective role of trehalose in the outstanding drought stress tolerance of *M. steppicola*
- determining the activity and inductive characteristics of trehalase isozymes, catalysing trehalose destruction, in the vegetative mycelia of *Morchella conica* and *M. steppicola*
- monitoring the main processes of trehalose metabolism (synthesis, accumulation, degradation) throughout the whole vegetative life cycle of *M. steppicola*
- determining the activity and induction characteristics of trehalase isozymes in vegetative and reproductive organs of *Populus glauca* L. var *Kopeczkii* and *Populus canescens*, in different phenophases
- monitoring the effects of biotic stress on the SOD, POD and trehalase isozyme activities in leaves of *P. glauca*
- isolation of the genes of trehalose metabolism from *M. steppicola*, *M. esculenta*, *P. glauca* and *Quercus robur*, designing primers for this work, based on the previously

published gene sequences of trehalase and the trehalose synthase enzyme complex, acquired from molecular biological databases (NCBI, EMBL-EBI)

### III. Materials and Methods

#### Materials

##### Fungal materials

*Morchella conica* Pers. and *Morchella steppicola* Zer. strains were isolated from young fruitbodies, and monospore-cultures were established from ascospores. Cultures were started on PDA- or MA-plates. For the maintenance of the strains and the experiments on trehalose metabolism, modified MS media were applied (Murashige and Skoog 1962), mycelia were cultivated at 23°C.

##### Plant materials

Trehalose metabolism was investigated in micropropagated poplar plants (*Populus glauca* L. var *Kopeczkii*) as well as in environmental samples of *Populus canescens* (Aiton)Sm. (grey poplar) and *Quercus robur* L. (pedunculate oak). Micropropagated plants were grown in hydroponic cultures, in dark-walled glass vessels, in ¼ Hoagland media (Hoagland and Arnon 1950).

##### Experimental designs

- A. Different experiments were conducted on fungal strains maintained both on solid and in liquid media. 3-5 parallel samples were analysed in each design.
  - 1. Monitoring the processes of trehalose metabolism (synthesis, accumulation (PS-formation) and degradation) during the vegetative life cycle of *M. steppicola*.
  - 2. Trehalase isozyme induction in the vegetative mycelium of *M. steppicola* and *M. conica*
  - 3. Investigation of drought stress tolerance of *M. steppicola*
- B. Experiments on micropropagated and environmental plant material
  - 1. Investigating the effect of biotic stress on the superoxide-dismutase, peroxidase and trehalase activity of micropropagated *Populus glauca* L. var *Kopeczkii* plants.
  - 2. Determining the activity of trehalase isozymes in vegetative and reproductive organs of micropropagated and environmental samples of *Populus canescens*, in different stages of development.
  - 3. Investigation on the inducibility of trehalase isoforms in mature *P. glauca* leaves. Leaves, detached non-destructively at their bases, were put into an Erlenmeyer flask

filled with  $\frac{1}{4}$  Hoagland medium with 3mM trehalose. Continuous illumination of  $150 \mu\text{E m}^{-2} \text{ s}^{-1}$ ,  $20^{\circ}\text{C}$  and 70 % relative humidity were provided.

- C. Identification of TPS and trehalase genes in the genomic DNA of *M. steppicola*, *M. esculenta*, *P. glauca*, *P. canescens* and *Quercus robur*.

## Methods

### Enzyme preparation

1. When preparing trehalase enzyme extract, mycelia were homogenized in two volume, resting bodies and plant materials were homogenized in three volume of TRIS-based extraction buffer (0.1 M, pH=7.50). Extracts were centrifuged (6000 g,  $4^{\circ}\text{C}$ , 10 minutes); the supernatant contained the isozymes.
2. For the determination of extramycelial trehalase activity, culture media were filtered through three layers of gauze, then the media were freezed ( $-20^{\circ}\text{C}$ ) and lyophilised ( $-40^{\circ}\text{C}$ ,  $10^5$  Pa, 48 hours; Jencons freeze dryer, Bedfordshire, England). Prepared samples were suspended in 25.0 mM TRIS-HCl buffer (pH=7.50) and dialysed in 10.0 mM TRIS-HCl overnight at  $4^{\circ}\text{C}$ .
3. When preparing SOD and POD enzymes, fresh mycelia were homogenized in two volumes, resting bodies were homogenized in three volumes of 25.0 mM Sörensen phosphate buffer (pH=8.00). Extracts were centrifuged (10000 g,  $4^{\circ}\text{C}$ , 15 minutes); the supernatant contained the isozymes.

### Determination of the total protein content

Total protein content of the enzyme extracts were determined with *Bradford's* method, with the application of BSA standard (Bradford 1976).

### Determination of enzyme activities

1. The activity of trehalase isozymes were determined by the spectrophotometrical measurement of glucose, released into the medium during the incubation.
2. For the determination of superoxide-dismutase (SOD) and peroxidase (POD) enzymes activities, isoforms were separated by polyacrilamide gelelectrophoresis (PAGE). In case of SOD isozymes modified SDS gradient PAGE, for the separation of POD isozymes 10% homogenous native PAGE system were applied. Subsequent to separation, isoforms were visualized by activity staining. Staining of SOD gels were accomplished by the method of *Beauchamp and Fridovich (1971)*, for POD electrophoretograms ortho-dianizidine containing solution was used. Stained gels were digitalized with a high-resolution scanner (HP ScanJet® 7400C, California,

USA), densitometry evaluation was accomplished with the program Phoretix™ 1D (Newcastle, United Kingdom).

#### Determination of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> content

O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> content of *M. steppicola* samples was determined according to *Belinky et al. (2003)*.

#### Determination of the dry weight and the MDA content of the samples

Dry weight of both plant and fungal samples were measured after incubating them at 50°C overnight in an exsiccator. Malondialdehyde (MDA) content was determined according to *Placer et al. (1966)*.

#### Determination of the pigment content

Chlorophyll content of plant materials was determined in 80% acetone with the method of *Porra et al. (Porra et al. 1989, Porra 2002)*. Total carotenoid content was quantified according to *Arnon (1949)* with the modification of *Lichtenthaler and Wellburn (1983)*. Anthocyanin content of the samples was determined with the method of *Sims and Gamon (2002)*.

#### Determination of glucose and trehalose content

Glucose and trehalose content of *M. steppicola* mycelia and resting bodies were determined in 80% ethanol extracts with high performance thin layer chromatography (HPTLC). Chromatograms were visualized with modified Antron® reagent. Following digitalization, densitometry was accomplished (HP ScanJet® 7400C, Phoretix™ 1D).

#### Isolation of genomic DNA, primer design

Total genomic DNA was isolated with the method of *Gawel and Jarret (1991)*. Precipitated DNA was dissolved in sterile, nuclease-free distilled water. DNA concentration and purity of the samples were determined with a spectrophotometer (A<sub>260</sub>, A<sub>280</sub>).

For the identification of TPS and trehalase genes of the studied fungal and plant species self-designed primers were applied. Based on the conservative regions of DNA and protein sequences, available from molecular databases, degenerate oligonucleotide primers were designed (CODEHOP, manual design).

#### PCR, fragment isolation, sequencing

Amplification was accomplished with a BioCycler TC-S (BIOSAN) device. Total volume of PCR mixture was 25 µl in the preliminary tests, and 50 µl subsequent to

the successful amplification. Final concentration of  $Mg^{2+}$  was 2.5 mM. Result of the PCR was checked by agarose gel electrophoresis in an Embi Tec RunOne™ system, stained with ethidium bromide. DNA fragments within the expected range of size were isolated back from the gel with Promega Wizard® kit (Madison, WI USA). Isolated fragments were dissolved in sterile, nuclease-free milliQ water. Sequencing reaction was accomplished with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), then the sequences were prepared for the electrophoresis (DNS precipitation with ethanol-containing Na-acetate solution, washing with ethanol, drying the precipitate). Capillary gel electrophoresis was carried out with an ABI Prism 3100 Genetic Analyser device (Applied Biosystems).

#### Sequence analysis

Similarities between the edited sequences and the published gene sequences of trehalose metabolism, assembled from international databases (GenBank, EMBL), were calculated by the BlastN software. DNA sequences were aligned with the ClustalW2 program.

### **IV. Results and Discussion**

#### Trehalose metabolism during the vegetative life cycle of *Morchella steppicola*

My results on the vegetative mycelium and resting bodies (PS) of *Morchella steppicola* revealed the relationship between the induction of resting body formation, trehalose synthesis and accumulation and oxidative stress, **I have proved the inductive role of oxidative stress in PS establishment**, a process that leads to trehalose accumulation in the mycelia zone of the forming resting body, reaching a maximum trehalose concentration by the maturity of the PS. Prior to the appearance of the PS gradually increasing oxidative stress can be detected in the respective mycelial zone, then it tends toward a resting state with the maturation process of  $PSI \rightarrow PSD \rightarrow PSM^*$ . I have monitored the presence and intensity of oxidative stress by measuring the changes in the SOD isozyme activity, the  $O_2^{\cdot-}$ ,  $H_2O_2$  and MDA content. My results indicate that **no trehalose is transported into the mycelium during PS establishment; rather it is synthesized locally, right in the place of utilisation**. My results on resting body forming and PS deficient *M. steppicola* strains, maintained on solid media of variable trehalose content, proved the inductive effect of external trehalose resource on the resting body formation of PS-forming mycelium. \**PSI – pseudosclerotium initial, PSD – developing PS, PSM – mature PS*

In addition to the synthesis and accumulation of trehalose, I have monitored the process of degradation throughout the whole vegetative life cycle of *M. steppicola*. Based on the activity profile of trehalase isoforms of different pH optima and localisation, two main trends can be delineated (activity trend<sub>1</sub>, activity trend<sub>2</sub>). During the PS establishment (activity trend<sub>1</sub>), in the PSI → PSD → PSM process, as well as during the germination, the activity of neutral mycelial trehalase is significantly higher than that of the acidic trehalase isoforms. Enzyme activity measured in the stages PSI – PSD – PSM decreases beneath the level of the mature (control) mycelia. Considerably elevated activities, which are characteristic of the beginning of germination, admirably decrease with the growth of the young cultures.

Subsequent to germination (activity trend<sub>2</sub>), during the growth and maturation of the mycelium, significant change was observed in the activity profile of mycelial trehalases: values became even. The lowest activity was measured in the pigmented, mature mycelia. The two trends can be delimited by the appearance of the secondary mycelium prior to the establishment of the resting bodies, when the activity of the acidic trehalase considerably increases. Among natural circumstances, this is the highest of the measurable values during the vegetative life cycle.

**These results demonstrate that beside neutral mycelial trehalase isoforms, acidic isozymes also play a considerable role in the regulation of endogenous trehalose concentration as well as in trehalose utilisation.**

#### Comparative analysis of trehalase activity of *Morchella steppicola* and *Morchella conica*

Comparative analysis of trehalase enzyme activity of *M. steppicola* and *M. conica* vegetative mycelia was carried out under inductive conditions. **My results demonstrated the inducibility of (extramycelial and excreted) neutral and acidic mycelia isoforms**, and also proved their role in the utilisation of external trehalose. **All along the induction process, activity of acidic isoforms exceeded that of the neutral isoforms**, and the activity values measured in the mycelia of *M. conica* were lower than those of *M. steppicola*.

#### Drought stress tolerance of *Morchella steppicola*

*M. steppicola*, adapted to extremely harsh environments, has extraordinary drought stress tolerance. My results indicate that bioprotective ability of trehalose has a significant role in this phenomenon. In artificially induced osmotic stress, triggered by polyethylene-glycol (PEG), changes in the activity of superoxide-dismutase isozymes, as



a part of the physiological response to the drought stress, is indicative of the stress affecting the mycelium and the activation of an effective defence system being in the background of the tolerance. **Parallel to the increasing degree of drought stress, considerably increased the trehalose content of the mycelia, with a gradual decrease of the proportion of glucose.** Due to its resistance potential, mycelium of *M. steppicola* can remain in the resistance stage of the stress syndrome even in case of severe osmotic stress (caused by 15-18 m/V % PEG), and only the highest applied concentration of PEG (21%) put them into the stage of exhaustion.

#### Trehalase activity of *Populus* species

**Different values of trehalase activity were detected among standard conditions in the vegetative and the reproductive organs of *Populus glauca* L. var *Kopeczkii* and *Populus canescens* (Aiton)Sm., characteristically with higher values in case of the generative organs.** Trehalose metabolism in plants influences the starch-sucrose metabolism, as well as the developmental processes, what is also supported by the results of my investigations carried out in the stage of budding.

*P. glauca* leaf experiments demonstrated the inducibility of trehalase enzyme, with the dominance of the neutral cytoplasmic isoforms. Activity of these enzymes gradually decreases with time.

#### Effect of biotic stress on the SOD, POD and trehalase activity of *Populus glauca*

Establishment and degree of the biotic stress was monitored in the leaves of *P. glauca* by detecting the changes of SOD and POD isozyme activity, pigment and MDA content. In *P. glauca* leaves, **biotic stress increased the activity of trehalase isoforms**, what is probably caused by the alteration of primary metabolism and the activation of plant defence mechanisms. **Activity values of acid trehalase isozymes always exceeded those of the cytoplasmic neutral isoforms. With the exaggeration of the stress activity of acidic isoforms decreased.**

#### Identification of genes of trehalase metabolism in the genomic DNA of *Morchella steppicola*, *Morchella esculenta*, *Populus glauca* and *Quercus robur*

With the application of self-designed primers, I have managed to identify **TPS and trehalase genes** in the genomic DNA of *M. steppicola*, *M. esculenta* and partial TPS genes in *P. glauca* and *Quercus robur*.

## V. Publications in the topic of the dissertation

### Papers published in referred scientific journals

Király, I. and **Czövek, P.** (2007): Oxidative burst induced pseudosclerotium formation of *Morchella steppicola* Zerova on different malt agar media. *Canadian Journal of Microbiology* 53(8): 975-982. (IF 1.275)

**Czövek, P.** and Király, I. (2011): Inducible trehalase enzyme activity of *Morchella conica* Persoon mycelium. *Acta Microbiologica et Immunologica Hungarica* 58(1): 1-11.

### Conference participations

#### Conference abstracts published in English

**Czövek, P.** and Király, I. (2002): The function of trehalose in the Morel life cycle. *Acta Microbiologica et Immunologica Hungarica* 49: 394-395. Second Hungarian Conference of Mycology, 29-31 May 2002 Szeged, Hungary

**Czövek, P.** and Király, I. (2005): Comparative assay of the drought stress tolerance of two Morel species (*Morchella steppicola* Zer. and *Morchella esculenta* L.). *Acta Microbiologica et Immunologica Hungarica* 52(2): 242-243. III. Magyar Mikológiai Konferencia, Mátraháza 2005. május 26-27.

**Czövek, P.,** Kálmán, G. and Király, I. (2008): The inducible trehalase activity in *Cenococcum geophyllum* Fries. *Acta Microbiologica et Immunologica Hungarica* 55(2): 180-181. IV. Magyar Mikológiai Konferencia, Debrecen 2008. május 29-31.

#### Conference abstracts published in Hungarian

**Czövek, P.** and Király, I. (2004): Sztyeppei kucsmagomba (*Morchella steppicola* Zer.) szárazságstressz-toleranciájának élettani vizsgálata. Magyar Mikrobiológiai Társaság 2004. évi Nagygyűlése Keszthely, Hotel Helikon 2004. október 7-9.

## VI. Further publications close to the topic of the dissertation

### Papers published in referred scientific journals

**Czövek, P.,** Király, I., Páldi, E., Molnár, I. and Gáspár, L. (2006): Comparative analysis of stress tolerance in *Aegilops* accessions and *Triticum* wheat varieties to detect different drought tolerance strategies. *Acta Agronomica Hungarica* 54(1): 49-60.

#### Full papers published in conference proceedings

Király, I. and **Czövek, P.** (2002): Changes of MDA level and O<sub>2</sub> scavenging enzyme activities in wheat varieties as a result of PEG treatment. *Acta Biologica Szegediensis* 46(3-4): 105-106. 7th Hungarian Congress on Plant Physiology 24-27 June 2002 Szeged, Hungary

#### Chapters in scientific books

Szücs, E., Balla, I., Kirilla, Z., Vörös, I., Takács, T., Király, I. and **Czövek, P.** (2008): Symbiotic and vitalizing effects of microorganisms in counterbalancing the replant disease of fruit trees. In: *Mycorrhiza Works* 2008: 248-257. (Feldmann F., Kapulnik Y., Baar J., eds.) ISBN 978-3-941261-01-3 © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

#### Conference abstracts

##### Conference abstracts published in English

**Czövek, P.** and Király, I. (2005): Phylogenetics of *Morchella steppicola* (Zer.), *Morchella esculenta* (L.) and *Verpa digitaliformis* (Pers.ex Fr.) based on nuclear ITS sequences. *Acta Microbiologica et Immunologica Hungarica* 52(2): 227. III. Magyar Mikológiai Konferencia, Mátraháza 2005. május 26-27.

#### Professional lectures given in scientific institutes

Király, I., **Czövek, P.** and Kálmán, G. (2007): „Ektomikorrhiza gombák és egyéb talaj-mikroorganizmusok vitalizáló hatásai talajunt és stresszkörülmények között.” Szimbionta és vitalizáló mikroorganizmusok az integrált gyümölcstermesztésben szimpózium, ÉGyDKF Kht., Budapest 2007. november 28.